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IDENTIFICATION OF A HYBRID LETHAL GENE ON THE X CHROMOSOME OF *CAENORHABDITIS BRIGGSAE*

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

By

JOHN KELLY DOUGHERTY
B.A., Miami University, 2016

2019
Wright State University

WRIGHT STATE UNIVERSITY

GRADUATE SCHOOL

5 December 2019

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY **John Kelly Dougherty** ENTITLED ***Identification of a Hybrid Lethal Gene on the X Chromosome of Caenorhabditis briggsae*** BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF **Master of Science.**

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ABSTRACT

Dougherty, John Kelly, Department of Biological Sciences, Wright State University, 2019. Identification of a Hybrid Lethal Gene on the X Chromosome of *Caenorhabditis briggsae*

Two closely related *Caenorhabditis* species, *C. briggsae* and *C. nigoni* are cross fertile and produce viable adult progeny. From *C. nigoni* mothers, F1 adult females are viable and fertile, F1 males are viable but sterile. In crosses that utilize *C. nigoni* males and *C. briggsae* hermaphrodites produce viable adult F1 females but F1 males arrest during embryogenesis. A mutation in the *Cbr-him-8* gene is a recessive maternal-effect suppressor of male-specific lethality. Hybrid crosses with *cbr-him-8* mutant mothers produce viable adult male progeny. The HIM-8 protein in *C. elegans* is required for the pairing of X-chromosomes during meiosis. This function is likely conserved in *C. briggsae.* Unpaired chromosomes are transcriptionally silenced in a wide variety of taxa. Based on this information it's been proposed that the meiotic silencing of unpaired chromosomes (MSUC) is suppressing an X-linked hybrid lethal gene responsible for male specific lethality. Multiple co-suppression assays identified two genes as candidate hybrid lethal genes, CBG30927 and CBG00239. These genes were knocked out with RNAi and CRISPR to evaluate if either of these genes were a hybrid lethal gene. sgRNA/Cas9 complexes and dsRNA of the candidate hybrid lethal genes was injected into *C. briggsae* hermaphrodites. Injections using CRISPR were able to disrupt expression of control targets but not the candidate hybrid lethal genes. Both RNAi and CRISPR injected nematodes were mated with *C. nigoni* males and the resulting progeny were scored for viable F1 males. From injections of dsRNA containing copies of CBG30927 or *Cbr-hig-1,* male progeny were derived. *Cbr-hig-1* has a syntenic ortholog in *C. nigoni* that is not present in any other *Caenorhabditis* species. Several regions

were identified in the *C. briggsae* and *C. nigoni* transcripts including an exon 5 extension that is responsible for a change in the predicted structure of the proteins that could be responsible for the dysgenic interactions.

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ACKNOWLEDGMENTS

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Introduction

Reproduction between organisms from segregated populations produce offspring that are less suited to survive. Reduced gene flow between separated populations over time allows for the accumulation of asymmetric incompatibilities within their genomes. (Dobzhansky *et al.,* 1936; Coyne *et al., 2004).* A segregated population would not necessarily have maintained the normal interactions of genes found in the original population and over time these differences deteriorate the compatibility of the organisms. The reduced fitness of hybrid offspring is the result of allele-specific dysgenic interactions that arise because of these differences (Coyne *et al.,* 2004). The gradual divergence of their genomes acts as a mechanism in the speciation of distinct populations assuming allopatric speciation without the influence of ecological factors. Speciation is the point that these differences add up to an inability of two populations to reproduce together (Maine *et al.,* 2010). The genetic mechanisms that maintain hybrid incompatibility prevent the generation of offspring or disable the offspring's ability to survive.

There are several mechanisms of hybrid incompatibility. They can occur both before and after embryogenesis (Bowring et al., 2005). Pre-zygotic isolation is the temporal, behavioral, and mechanical mechanisms while post-zygotic isolation describes all isolation that acts after fertilization and can have heritable regulatory consequences. Most of what is known about post-zygotic isolation comes from studies of *Drosophilla* hybrids. The DNA binding protein *lhr* was one of the first genes identified as responsible for the post-zygotic isolation of *Drosophila melanogaster and D.simulans* (Maheshwari et al., 2012). The genetic basis for the post-zygotic isolation in *Drosophilla* is the result of *lhr*'s negative ectopic interactions across several loci that are directly responsible for hybrid sterility and inviability (Orr and Irving, 2001; Wirrbrodt et al.1989; Ting et al.,

1998). While the mechanism for post-zygotic isolation has been characterized in *Drosophila*, not all of what we learned can be generalized.

Another known reproductive isolation mechanism exists in *Caenorhabditis* and are generally post-zygotic and produce dead or sterile hybrids (Baird and Seibert, 2013). Unlike in *Drosophila*, the gene(s) and mechanism(s) responsible have not yet been characterized. In *Caenorhabditis* two species pairings, *C. remanei* to *C. latens* and *C. briggsae* to *C. nigoni* are able to produce hybrids (Dey et al., 2012; 2014). In addition to hybrid crosses featuring *C. briggsae* and *C. nigoni* described previously, the hybrid lethality observed in *C. latens* produces fertile F1s and F2s experience hybrid breakdown. Crosses featuring *C. remanei* produces sterile F1 females with abnormal gonads. Typical hybrid crosses between *C. briggsae* mothers and *C. nigoni* fathers result in a male specific lethality. While these species are cross fertile and produce viable F1 adults, the reproductive crosses between *C. briggsae* males and *C. nigoni* females produce fertile adult females and infertile adult males. Crosses between *C. briggsae* hermaphrodites and *C. nigoni* produce viable female progeny, however male hybrids are arrested during embryogenesis (Woodruff *et al.,* 2010). The male-specific lethality observed from this reciprocal cross is likely the result of dysgenic interactions between an X-linked gene in *C. briggsae* and other autosomal genes within the *C. nigoni* genome (Bi et al., 2015; Ragavapurm et al., 2016).

F1 male specific lethality observed in hybrid crosses between *C. briggsae* and *C. nigoni* is suppressed by the *cbr-him-8(v188)* mutation (Ragavapurm et al., 2016). In crosses featuring *cbr-him-8* mutant *C. briggsae* hermaphrodites, 18% of progeny are F1 males that are viable into adulthood. Additionally, the percentage of males produced in crosses featuring heterozygous *cbr-him-8* organisms is consistent with a maternal effect suppression of male-specific lethality. The result of a knockout of the HIM-8 protein in *C. elegans* is X-chromosome nondisjunction (Hodgkin et al., 1979): X-chromosomes that

fail to pair properly during meiosis are traditionally transcriptionally silenced. Meiotic silencing of unpaired chromosomes is a piRNA mediated process that targets heterogametic sex chromosomes, such as the X chromosome of male nematodes, as well as any other chromosomes that fail to synapse properly due to mutation (Kelly et al., 2002). The main function of MSUC is epigenetic control of embryogenesis (Maine et al., 2010). Regions of the chromosome without active synapsis during prophase are marked with repressive histone modification. Dimethylation of histone 3 on lysine 9 triggers the activation of the piRNA pathway that recruits the suppressors of the entire portion of the chromosome. When this occurs on the *C. briggsae* X-chromosome in *cbr-him-8* mutants, it appears that a gene responsible for hybrid lethality is suppressed (Ragavapurm et al., 2016).

Based on this model, a co-suppression assay was constructed by a previous graduate student to map the *C. briggsae* X-chromosome for the male-specific hybrid lethal gene (Bittorf, 2018). In this assay, a number of transgenic strains were constructed that possessed bacterial chromosomal (BAC) derived extrachromosomal arrays. These BAC were composed of different segments of the X-chromosome that were previously associated with hybrid lethality (Bi et al., 2015). The extrachromosomal arrays derived from these BACs will be silenced during meiosis, as will the endogenous copies of any genes they contain. These strains were crossed to *C. nigoni* males and progeny was scored for viable F1 males. If BAC clones rescued F1 males, it was assumed that the BAC contained a hybrid lethal gene.

Two BACs from this assay were found to produce males (Bittorf, 2018). 17D03 produced F1 males at a frequency of 0.025. 08G05 produced males at a frequency of 0.130. Within the region of the *C. briggsae* X-chromosome covered by 08G05, there were 11 predicted protein coding genes. The 11 candidate hybrid lethal genes were the target of subsequent co-suppression assays utilizing PCR product mixtures. The PCR

product mixtures of the candidate hybrid lethal genes were split between 4 different groupings that were injected into *C. briggsae* hermaphrodites. These groupings were odd numbered, even numbered, right portion, and left portion of the candidate genes within a series as they are predicted to appear on the X-chromosome. Based on the phenotypic results of each injection group, CBG30927 and CBG00239 are the most likely to be the hybrid lethal gene.

In order to assess if these genes are hybrid lethal genes, they were targeted for silencing with multiple methods. Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) knockouts of the candidate genes were first used to screen for suppression of the hybrid lethal gene. Suppression of the male-specific lethality would indicate which gene is responsible for the reproductive barrier. Next I performed RNAi-mediated silencing of these genes on P0 *C. briggsae* hermaphrodites. I identified CBG30927, which is now designated *Cbr-hig-1* as for *C. briggsae* ***h****ybrid* ***i****ncompatibility* ***g****ene 1*.

Specific aims

Aim 1. To identify a hybrid lethal gene on the *C. briggsae* X-chromosome that is involved in F1 male-specific lethality.

Aim 2. To compare the *C. briggsae* hybrid lethal gene to its *C. nigoni* ortholog. Differences between these genes are candidates for evolutionary changes involved in the dysgenic interactions that cause F1 male-specific lethality.

Aim 3. To characterize the evolution of the *C. briggsae* hybrid lethal gene and its *C. nigoni* ortholog.

Materials and Methods

Nematode strains and maintenance

The *C. briggsae* AF16 strain was obtained from the *Caenorhabditis* genetics research center. The *C. nigoni EG5268* strain was provided by Marie-Ann Félix. Nematodes were grown on agar plates of *Escherichia coli* strain DA837.

The agar plates used were created by adding 18 grams of powdered agar and 5.9 grams of worm nutrient per 1 L of agar into 1000 ml flasks. The worm nutrient was weighed then placed within a beaker and mixed with 500 ml of Milli-Q water. Once the worm nutrient had dissolved the 500ml of Milli-Q water was added to a 1000 ml graduated cylinder containing another 500ml of Milli-Q water. 500ml of this mixture was separated into two 1000 ml flasks. 9 ml of agar powder would then be added to each flask and then the solution was autoclaved for 75 minutes with a 30-minute liquid cycle. The agar was then cooled for 20 min in a warm water bath. During that time period 2.5 ml of Streptomycin was added to the solution. The agar was then aliquoted into 60 mm petri dishes

Microscopy

Nikon microscopes were utilized for routine procedures and nematode strain maintenance. A Zeiss axiovert 35M microscope set at 400X magnification was used for the Microinjections. A World Precision Instrument vertical puller was used to create the needles used for microinjection and the reagents were loaded using drawn out Pastuer pipets. Injections were driven by compressed air at 35 psig.

Reagents

Primers used to inactivate the target genes via RNA interference were synthesized from primers using New England Bio's HiScribe™ T7 High Yield RNA Synthesis Kit. Reactions were synthesized at room temp and incubated at 37 degrees Celsius for an hour. dsRNA was purified by a spin column and the concentration was

verified by a nanodrop spectrophotometer at around 1 uM-10 uM. The primers designed to produce the sgRNAs used to target the CRISPR sites were dissolved to produce a 1 µg/ml concentration in tris 8.0 pH and mixed with the sgRNA enzyme mix and buffer and heated to 37º C for 30 minutes. The sgRNA were then added to a solution of Cas9 with the associated buffer and kept at 37º C for an additional 30 minutes.

Lysis of nematodes for polymerase chain reaction (PCR) was accomplished by utilizing the Nematrix worm lysis kit. Buffers were combined and cycled between 75º C and 95º C for 15 minutes. DNA extraction was confirmed by gel electrophoresis, combining the DNA with a dye and running it through a 2% agarose gel. The extracted DNA was then PCR amplified using a 12.5ul of New England biolab's PCR master mix combined with 2.5 ul of the extracted DNA, 5ul of the PCR primers and 5ml of sterilized water. The results were also confirmed by gel electrophoresis.

CRISPR Knockouts

CRISPR knockouts of the candidate genes were generated and used to screen for loss of the male-specific lethality. CRISPR can be utilized to induce a deletion because of CRISPR-Cas9's ability to perform as a site-specific endonuclease. The short guide RNA (sgRNA) component of the construct can be used to identify a specific 20 nucleotide sequence. At first, two sites were targeted for each gene within the coding region. Subsequent injections featured a single CRISPR target site. A co-CRISPR target confirmed the efficacy of the microinjection. A readily identifiable phenotype, as was the twitching produced when cbr-*unc-22* is silenced, was selected to visually confirm the success of the injection. *cbr-unc-22* was particularly useful because a heterozygous deletion could be identified by submersion of the nematode in a solution of 1% nicotine. An additional benefit was that the rates of success between injections was used as a benchmark to compare the efficacy the CRISPR designed for the target.

The efficacy of a CRISPR knockout for *cbr-unc-22* was established by calculating observed versus expected knockout based on successful microinjections. The progeny of the twitching nematodes were lysed and then polymerase chain reaction (PCR) amplification of the edited *cbr-unc-22* gene was used to confirm the knockout. The edited gene's amplicon would be noticeably shorter than the wild type on an agarose gel after gel electrophoresis. Within nematode strains that successfully produced *cbr-unc-22* knockouts, the F2 generation was screened for suppression of male specific lethality by mating sperm depleted F1 hermaphrodites with *C. nigoni* males. An F2 male produced by that cross would identify the target gene as a hybrid lethal gene. The knockout of the target genes within strains that produced males would then be confirmed by PCR amplification of the target sequence and comparing the change in the product size to the original gene with gel electrophoresis.

Synthesis of dsRNA

Another method for silencing the target genes utilized dsRNA which causes endogenous gene silencing via RNAi. dsRNA were generated from gene-specific PCR products that included T7 RNA polymerase promoter sequences at both ends. dsRNA was produced using T7 RNA polymerase (New England Biolabs, M0251S). Primer pairs used for gene specific amplifications for were **taatacgactcactatag**cggatgctgaaaaagcaaa & **taatacgactcactatag**aatacctctcggggcaactt for CBG00239 and taatacgactcactataggccacctcttgcagtcctac & **taatacgactcactatag**tgaagcagatagctcccgat for CBG30927. The T7 promoter sequences in these primers are indicated in bold. *C. briggsae* AF16 hermaphrodites were with dsRNA as young adults, allowed to recover and then mated to *C. nigoni* EG5268 males. F1 progeny were scored for the presence of viable hybrid males.

Injections and Crosses

Adult AF16 *C. briggsae* hermaphrodites were loaded onto a 1% agar pad that had been dehydrated for 1 hour. The loaded agar pad was then placed under the Axiovert microscope at 400X magnification. After the needle was filled with Cas9 sgRNA or dsRNA, it was positioned over one arm of the distal gonad and the microscope stage was moved to driven the needle tip into the gonad. Injections were driven by compressed air at 35 psig. Post-injection, nematodes recovered on a seeded agar plate for approximately an hour before male *C. nigoni* nematodes were placed on the plate. F1 animals were screened for male rescue.

Results

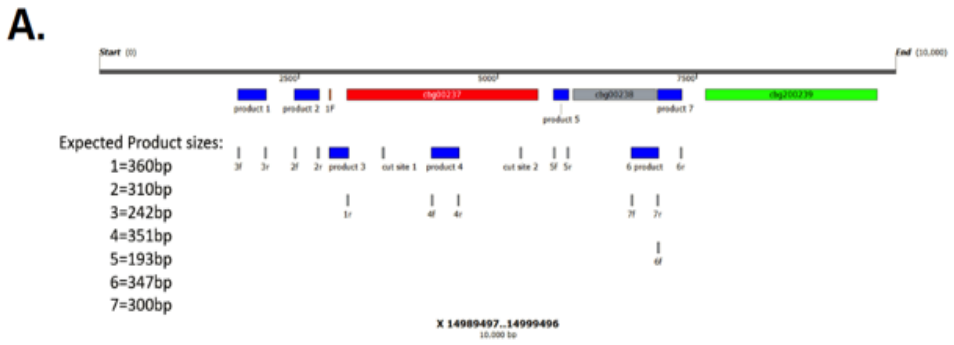
Aim 1: Identification of a *C. briggsae* hybrid lethal gene

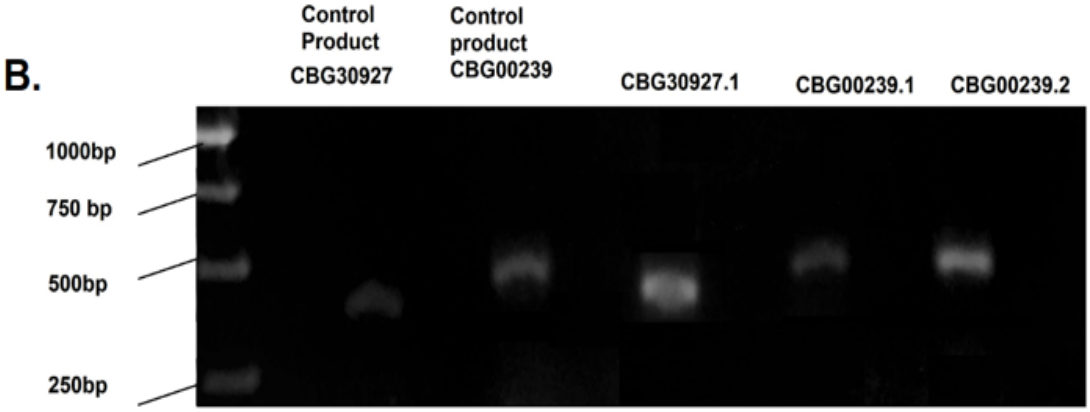
Of the eleven predicted protein-coding genes identified by Bittorf (2018), CBG30927 and CBG300239 were examined as the most likely hybrid lethal gene. In order to assess this, two approaches were utilized: gene mutations by CRISPR/cas9 nucleases and maternal gene silencing by RNAI.

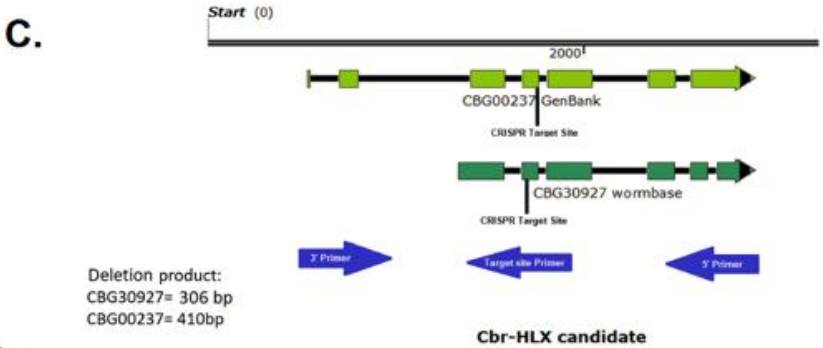
CRISPR/Cas9

Injections utilizing CRISPR were able to successfully produce the silenced *cbr-unc-22* phenotype. Multiple F1s were produced that readily twitched when 1% nicotine was applied indicating a heterozygous knockout. There were F2 progeny that twitched without prompting which indicated a homozygous knockout of *cbr-unc-22*. Several primers were designed to evaluate when PCR amplified, if a knockout had occurred, and how long a deletion might have taken place. The design of the primers made to ascertain this is depicted below (figure 1A). 28 injections that produced twitching nematodes failed to display any change in the length of either target sequence (table 1A). Gels run with the PCR products of these genes failed to display the change in size expected based on the design of the sgRNAs (figure 1B).

The two sgRNAs made to target each gene were designed to remove a 500-600bp portion of the genome between each sgRNA target. It was possible that a large deletion of the target gene would always produce a fatal outcome. This could explain how the endogenous silencing of the portions of the X-chromosome caused by the extrachromosomal arrays would produce males. Transcriptional silencing mediated by siRNAs produced by the injection of BACs could still produce the hybrid male viability while a large deletion of the gene caused by the CRISPR endonucleases might be fatal to the nematode. To that end, a new sgRNA target was chosen to create a smaller


A.
Start (0)
End (10,000)
2500
5000
7500
product 1
product 2
1F
product 5
product 7
Expected Product sizes:
1=360bp
2=310bp
3=242bp
4=351bp
5=193bp
6=347bp
7=300bp
3f
3r
2f
2r
product 3
cut site 1
product 4
cut site 2
5f
5r
6 product
6r
1r
4f
4r
7f
7r
6f
X 14989497..14999496
10,000 bp


B.
Control Product CBG30927
Control product CBG00239
CBG30927.1
CBG00239.1
CBG00239.2
1000bp
750 bp
500bp
250bp


C.
Start (0)
2000
CBG00237 GenBank
CRISPR Target Site
CBG30927 wormbase
CRISPR Target Site
5' Primer
Target site Primer
5' Primer
Deletion product:
CBG30927= 306 bp
CBG00237= 410bp
Cbr-HLX candidate

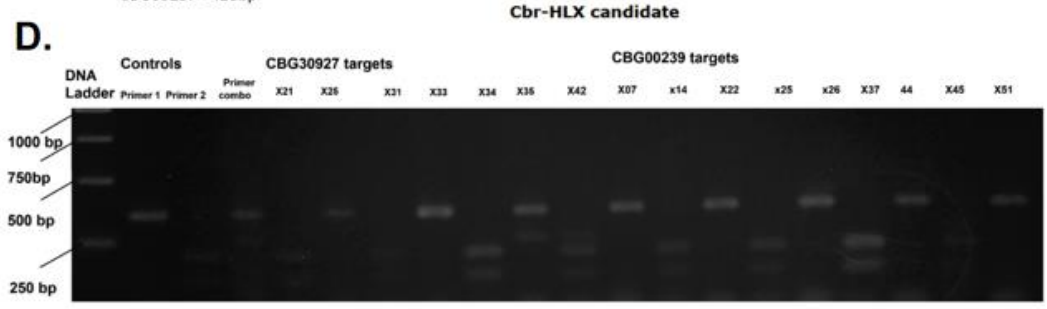

D.
Controls
CBG30927 targets
CBG00239 targets
DNA Ladder
Primer 1
Primer 2
Primer combo
X21
X26
X31
X33
X34
X36
X42
X07
x14
X22
x25
x26
X37
44
X45
X51
1000 bp
750bp
500 bp
250 bp

Figure 1. The designed PCR assay of sites targeted by CRISPR cas9 endonuclease for editing to ascertain the success of the gene knockout. (A) Several different sites amplified by PCR to confirm the knockout of both target sites in each gene by a change in product size. (B) A summary gel that reflects that a gene knockout by CRISPR cas9 was not observed. Product sizes did not change compared to the wild-type product. (C) The set-up of the second attempt to generate a CRISPR knockout and to evaluate that knockout. A primer was designed for the single target site utilized for each gene. Flanking primers would amplify two different products if the target site remained intact and a single large product if the target site had been removed. (D) Gels obtained from this assay produced many different and unexpected products that prevented the conclusive evaluation of a gene knockout.

Table 1. Recorded results of CRISPR/cas9 injections

two target sites	**Injections**	***unc-22* knockouts**[1]	**CBG knockouts**
CBG30927	480	96	0
CBG00239	364	58	0
single site targets	**Injections**	***unc-22* knockouts**[1]	**CBG knockouts**
CBG30927	104	12	0
CBG00239	104	16	0

[1] Identified in F1s by twitching phenotype in 1% nicotine.

break. Non-homologous end joining that would result from a break in the gene by the endonuclease that the sgRNA recruits could still reasonably knock down the gene since the large deletion proved fatal.

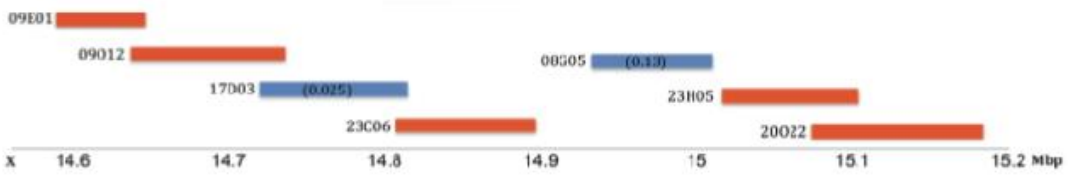
In order to evaluate a knockout, the PCR screen needed to be altered because the change in the genes size from the wild type were not big enough to be easily distinguished on a gel (figure 1C). So instead three PCR primers were used. One primer on either side of the gene facing opposite directions and one primer directly over the site targeted for endonuclease. If a knockout did not occur, PCR would amplify the middle primer and two products would be produced. If an edit occurs at the target site, the middle primer would not amplify and only one large product would be produced. This design also failed to display a deletion in the target genes among 154 successful injections (figure 1D). F1 self-progeny that were crossed with *C. nigoni* did not produce any males (table 1). The nematodes that were edited by the CRISPRs did not survive to the F2 generation were the PCR screening was taking place

Maternal RNAi-Induced Silencing

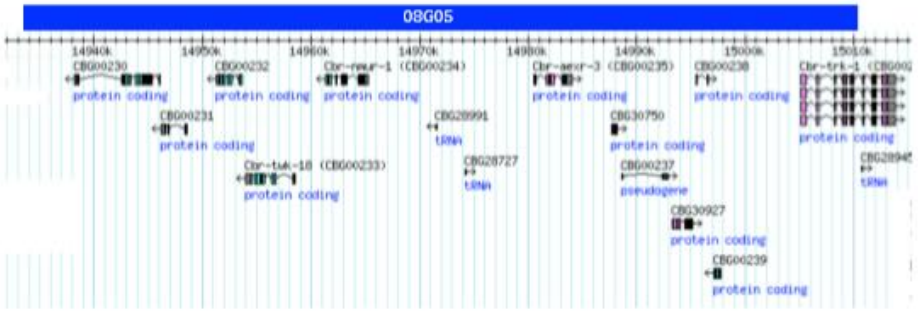
As an alternative, RNAi could solve many of the potential problems that CRISPR presented. RNAi acted at the transcript level and does not require genome editing. The piRNA mediated process is like the maternal effect suppression observed in Ragavapuram et al., and might closer replicate the results. The experimental design featuring RNAi was injected into the gonad in a similar way that did not require additional training to perform.

Injections utilizing this new methodology produced male progeny among F1 hybrids produced by *C. briggsae* hermaphrodites injected with CBG30927 dsRNA (figure 2). dsRNA was produced from dsDNA primers designed for a T7 polymerase depicted below (table 2). Nematodes injected with dsRNA from CBG300239 as well as

A.


09E01
09012
17D03
(0.025)
08G05
(0.13)
23H05
23C06
20D22
X
14.6
14.7
14.8
14.9
15
15.1
15.2 Mbp

B.


08G05
protein coding
pseudogene
tRNA

C.

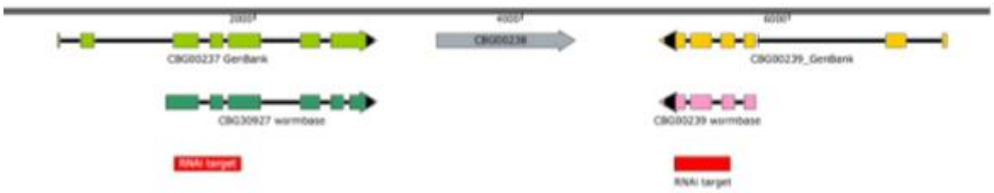

CBG00237 GenBank
CBG00238
CBG00239_GenBank
CBG30927 wormbase
CBG00239 wormbase
RNAi target
RNAi target

Figure 2. Genes within the region of the X-chromosome examined for the hybrid lethal gene. (A) This region was identified as associated with the suppression of male specific lethality mapped by BAC-derived extrachromosomal arrays by a previous graduate student. BACs that rescued F1 mal viability are depicted in blue and unsuccessful BACs are indicated in red. (B) Genes contained within the rescuing BACs with the highest efficacy of suppression. (C) Depicts the candidate hybrid lethal genes that were targeted for suppression.

Table 2. Primers used to make dsRNA used in RNAi[1].

Target	Primer	Sequence[2]	Product Size[3]
CBG30927	SEB139	**taatacgactcactatag**gccacctcttgcagtcctac	500 bp
CBG30927	SEB140	**taatacgactcactatag**tgaagcagatagctcccgat	
CBG00239	SEB141	**taatacgactcactatag**cggatgctgaaaaagcaaa	630 bp
CBG00239	SEB142	**taatacgactcactatag**aatacctctcggggcaactt	

[1] dsRNA injected at concentrations ranging from 1 – 10 ng/µl.
[2] T7 RNA polymerase promoter sequence indicated in **bold**.
[3] Product size expected from amplification of genomic DNA.

Control injections produced no male progeny. 125 male progeny were observed from the CBG30927 silenced hybrid cross out of a total 692 progeny at a rate of 18% (table 3). This is similar to the 10% male progeny derived from the *cbr-him-8* crosses. This identifies CBG30927 as a hybrid lethal gene. Accordingly, CBG30927 has been named *Cbr-hig-1*(*hig* = ***h****ybrid* ***i****ncompatibility* ***g****ene*).

F1 males obtained with *C. briggsae* X-chromosomes are fertile (table 4). That fertility depends on which genotype they were crossed with. When crossed with *C. nigoni* or F1 hybrids derived from *C. nigoni* females, no viable progeny or eggs were observed. When crossed with *C. briggsae* females, progeny was obtained including male progeny at a frequency of 38%. When crossed with F1 hybrids derived from *C. briggsae* females, most crosses resulted in no progeny; however, 1 cross did produce some progeny and at least two adult males. This result is similar to crosses of F1 males derived from *cbr-him-8* mutant females (Ragavapuram et al., 2016).

Table 3. RNAi Injection results.

	Buffer Control	**CBG00239**	**CBG30927**
# of injected animals	10	15	20
concentration of dsRNA (ug/ml)	0	10	10
F1 progeny scored[1]	2056	792	692
F1 males observed[1]	0	0	125

[1] Injected P0 *C. briggsae* AF16 hermaphrodites were allowed to recover and then were mated to *C. nigoni* EG5258 males. F1 hybrid progeny from injected hermaphrodites were scored for viable males. As injected hermaphrodites were wild-type, self-progeny were expected to all be XX hermaphrodites. Therefore, any male progeny observed were assumed to be F1 hybrids.

Table 4. RNAi crosses.

cross	cross results	female progeny	male progeny	male frequency
F1 males x *C. nigoni* EG5268 females	no viable progeny	-	-	-
F1 males x *C. briggsae* AF16 'females'[1]	viable adults	419	245	0.369
F1 males x $F1^{Cni}$ females[2]	no viable progeny	-	-	-
F1 males x $F1^{Cbr}$ females[3]	viable adults (one cross)	216	2	0.009

[1] Sperm-depleted hermaphrodites.

[2] F1 females derived from *C. nigoni* mothers.

[3] F1 females derived from *C. briggsae* mothers.

Aim 2: Comparisons of Cbr-hig-1 to Cni-hig-1

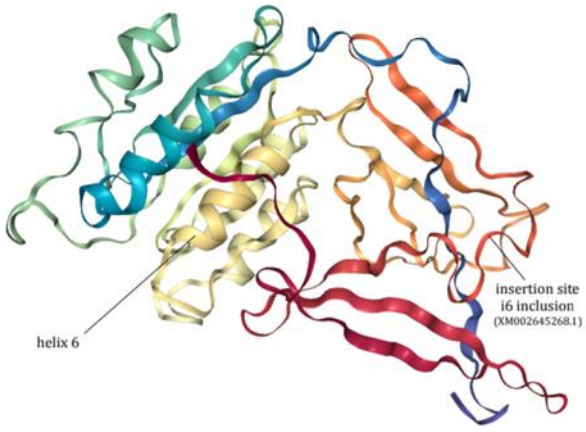
The next step after the identification of *Cbr-hig-1* as a hybrid lethal gene was to compare *Cbr-hig-1* to its orthologs and analyze its structure to ascertain what characteristics produce the dysgenic interactions that lead to hybrid lethality.

Predicted Protein structures

Secondary structure predictions of CBR-HIG-1 and CNI-HIG-1 matched the solved structures or alignment clusters of several proteins (figure 3). Secondary structures predicted for CBR-HIG-1.2 and CBR-HIG-1.3 were similar to CBR-HIG-1.1. Alpha helices 1-5 of CBR-HIG-1.1, CBR-HIG-1.2, and CBR-HIG-1.3 show conservation within their alignments and differ in sequences of alpha helix 6. The differential splice site usage corresponds with the gap in alignment at the position of helix 6. This is the same site that results in a 5' extension of exon 5 in all *cni-hig-1* transcripts.

Amino acids derived from the inclusion of intron 6 in CBR-HIG-1.2 correspond to gaps in the sequences of CBR-HIG-1.1 and CNI-HIG-1.4. The alignment clusters included structures from von Willebrand type A proteins (vWA) from *Catenulispora acidiphila* and *Sulfolobus acidocaldariu*s. vWA domains are typically involved with the formation of multiprotein complexes (Whitaker & Hynes, 2002). Additional alignment clusters were derived from human SEC23 & SEC 24, both of which contain vWA domains and the vWA domain of the p44 subunit of TFIIH

A.



B.

```
CBRHIG1.1   ----------------------NQKYFSDVKILKFCKYFFLDATSCSPTTEPQTFLFAYSNDFSSKTVLDTWKIFSTSPTFNSFYGSVRF 68
CBRHIG1.2   MQNNSDIGLMVRDENLMILREIVFGVWDKNSKHPSKSRRRPNATSCSPTTEPQTFLFAYSNDFSSKTVLDTWKIFSTSPTFNSFYGSVRF 90
CNIHIG1.3   ------------------MFKKNVPIIFLTKLAIVRILVVYDDTSCSPTTEPQTFLFAYSNDFSSKTVLDTWKAFSTLPTFNSFYGSVRF 72

CBRHIG1.1   DRDNLDIQFHTNIRNVNSTIAKNLPNSNQGFQNSSIFSNVFDAIEKFFSNTQAPVCGSIILILLKRYPNEADSSRLVSIIRSHHAIVHVI 158
CBRHIG1.2   DTDNLDIQFHTNIRNVNSTIAKNLPNSNQGFQNSSIGSNVFDAIEKFFSNTQAPVCGSIILILLKRYPNEADSSRLVSIIRSHHAIVHVI 180
CNIHIG1.3   DTENLDIHFNTNIRKVNSTIANDLPNPNQGFENSTTGSNAFDAIEKFFNNTQAPVCGSIILILLKRYPNEADSSRLVSIIRSHHAIVHVI 162

CBRHIG1.1   TSSTPSGGSQSQAMYSVTTKTNGMGAFEPDENFWD----------------------AIGWRPISGTLYPVYATTIQVSGKGKKTLPD 224
CBRHIG1.2   TSSTPSGGSQSQAMYSVTTKTNGMGAFEPDENFWD----------------------AIGWFPISFTLYPVYATTIQVSGKGKKTLPD 246
CNIHIG1.3   TSSTPSRGSQTKAMYSVATRTNGMGAFEPDEHFWDYYLKDIGDAFKKKKKKLPTGIFIFQAVGWFPISGTLYPVYATTIQVSGKGKKTLPD 252

CBRHIG1.1   FYPSITGAYLIALTYQDHVPDNSFRNFNLHFVNPGDSNFKGYQDGSSDL----------------LWDDGNYIAQAFWFLNLDYKMTLNYN 299
CBRHIG1.2   FYPSITGAYLIALTYQDHVPDNSFRNFNLHFVNPGDSGNFKGYQDGSSDLWVIFKNHSIRKFMISQDDGNYIAQSFWFLNLDYKMTLNYN 336
CNIHIG1.3   FYPSITGSYLIAITYQDHVPDNSFRSFNLHFFNPGDSGNFKSYPHGSSD----------------LWDDGNYIAQSFWFLNLDYKMTLDYN 327

CBRHIG1.1   YMGQDVQNLQIRIYSPFATNNWLPYND---- 326
CBRHIG1.2   YMGQDVQNLQIRIYSPRATNNWLPYND---- 363
CNIHIG1.3   YSGHNVQNLQIRVYSSFESSNWLPYND---- 354
```

Figure 3. Predicted structures of CBR- and CNI-HIG-1. (A) The predicted secondary structure of CBR-HIG-1.1. Similar structures were obtained for the other potential transcripts. (B) The alignment of CBR-HIG-1.1, CBR-HIG-1.2, and CNI-HIG-1.1 showing conservation of alpha helices 1 through 5 and the differing splice site usage that results in the 5' extension of exon 5 in all cni-hig-1 transcripts (figure 6). Amino acids derived from the inclusion of intron 6 (boxed) in CBr-HIG-1.2 correspond to gaps in the sequences of CBG-HIG-1.1 and CBR-HIG-1.3. Protein structure predictions were made by Robertta and Phyre2 servers (Kim et al., 2004, Kelly et al., 2015). Predicted alpha helices are highlighted in yellow and predicted beta strands are highlighted in blue. Sequences derived from parasite.wormbase.org and GenBank.

Aim 3: Evolutionary history of Cbr-hig-1

Cbr-hig-1 is within the region of the X-chromosome between the *cbr-aexr-3* and *cbr-trk-1* (figure 4). There is a syntenic ortholog of *CBG30927* in *C. nigoni*. However, it is absent from *C. sinica* and from all other species in the elegans group (figure 5). This indicates that these genes arose from an insertion in this region of the genome in the most recent common ancestor of *C. briggsae* and *C. nigoni*. It's not clear where this insertion came from, but, regardless of the source of that insertion, the divergence of the *hig* orthologs within the most recent common ancestor of *C. briggsae* and *C. nigoni* have resulted in the development of *Cbr-hig-1* into a hybrid lethal gene.

There are multiple transcripts that have been reported for both syntenic *hig* orthologs *Cbr-hig-1 and cni-hig-1* (figure 6). All these transcripts are largely co-linear at their 3' ends from exon 2-8. They differ somewhat in splice site usage and specific exon changes. There is an exclusion of exon 2 from both the *cni-hig-1.1* and the *cni-hig-1* predicted transcript. Exon *5* in the *C. nigoni* transcripts feature a 5' extension due to a difference in splice site usage between *C. briggsae* and *C. nigoni.* Additionally there is an exclusion of exon 8 from *cni-hig-1.2* and the inclusion of intron 6 in *Cbr-hig-1.2.* These transcripts were derived from Wormbase and GenBank, but these transcripts appear to be incomplete based on the fact that the Wormbase prediction for *Cbr-hig-1* appear to lack a start codon.

There are several paralogs of *Cbr-hig-1* and *cni-hig-1* within the genomes of *C. briggsae* and *C. nigoni* (figure 7). Cnig_g3391.1 is the most closely related at a 0.96 branch support aLRT overlap paralog based on the inferred protein sequences in the regions of overlap. Among the paralog transcripts, the *cni-hig-1* paralogs displayed a longer exon 5. One splice variant in *Cbr-hig-1* retained intron 6, which is consistent with these genes being derived characters. All paralogs depicted did not contain the exon 4 extension that was observed in the prediction for *cni-hig-1.*

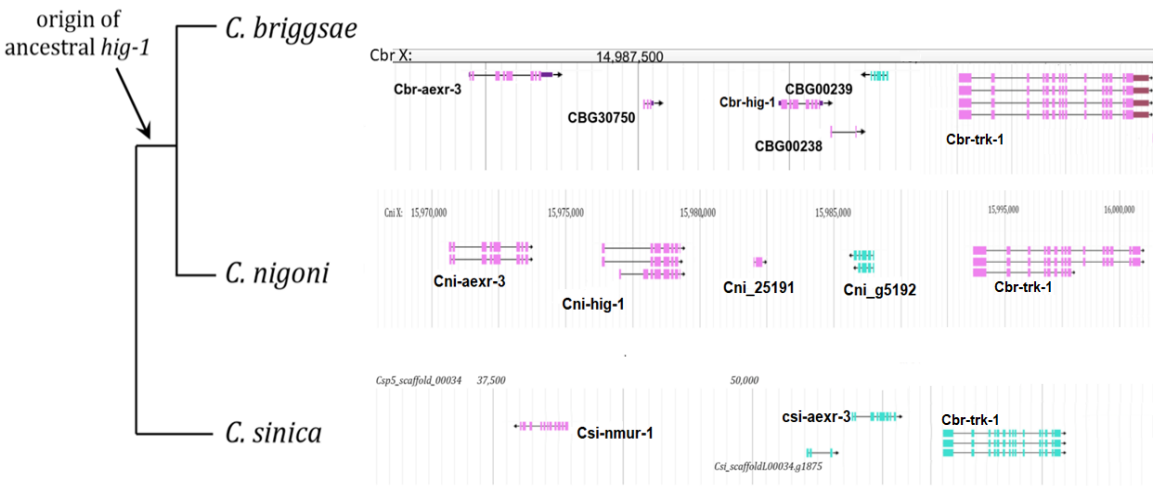

origin of
ancestral hig-1
C. briggsae
C. nigoni
C. sinica
Cbr X: 14,987,500
Cbr-aexr-3
CBG30750
Cbr-hig-1
CBG00239
CBG00238
Cbr-trk-1
Cni X: 15,970,000
15,975,000
15,980,000
15,985,000
15,995,000
16,000,000
Cni-aexr-3
Cni-hig-1
Cni_25191
Cni_g5192
Cbr-trk-1
Csp5_scaffold_00034 37,500
50,000
Csi-nmur-1
csi-aexr-3
Cbr-trk-1
Csi_scaffold1.00034.g1875

Figure 4. Regions within the X-chromosomes of *C. briggsae, C. nigoni* and *C.sinica* that share syntony between *aexr-3* and *trk-1* orthologs.

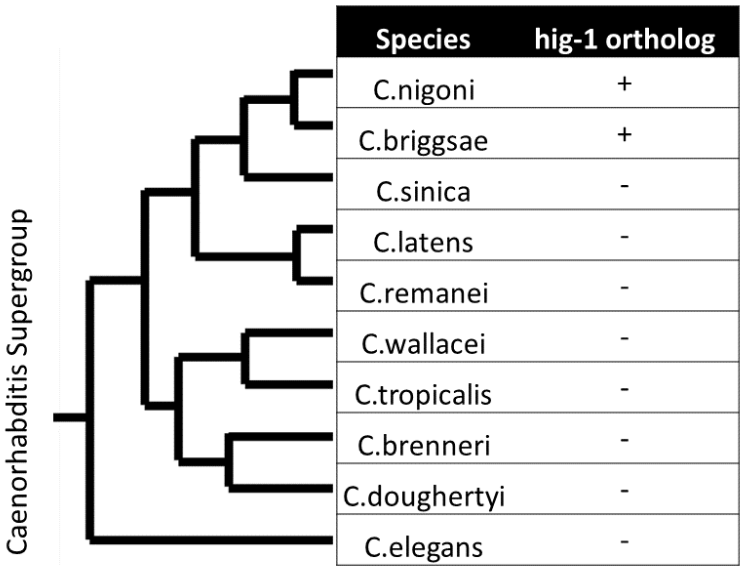

Caenorhabditis Supergroup
Species
hig-1 ortholog
C.nigoni +
C.briggsae +
C.sinica -
C.latens -
C.remanei -
C.wallacei -
C.tropicalis -
C.brenneri -
C.doughertyi -
C.elegans -

Figure 5. The species within the *Caenorhabditis* supergroup that contain a *Cbr-hig-1* ortholog.

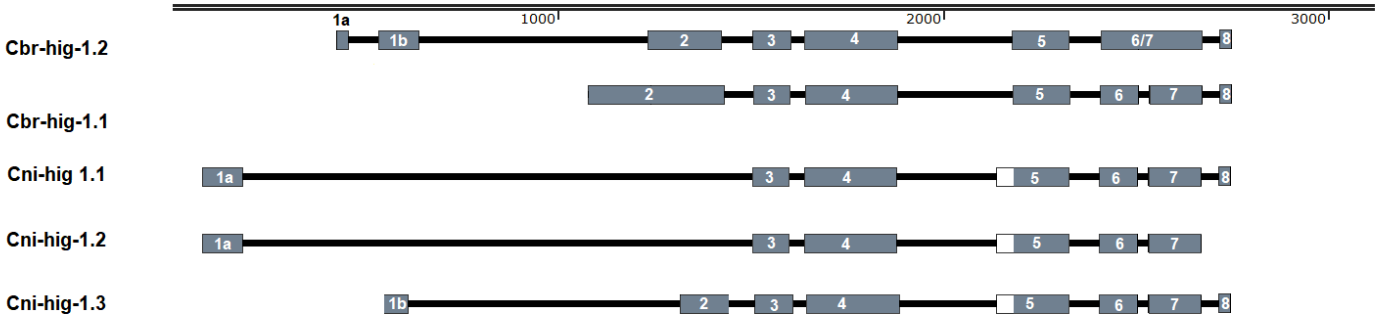

1a
1000
2000
3000
Cbr-hig-1.2
1b
2
3
4
5
6/7
8
2
3
4
5
6
7
8
Cbr-hig-1.1
Cni-hig 1.1
1a
3
4
5
6
7
8
Cni-hig-1.2
1a
3
4
5
6
7
Cni-hig-1.3
1b
2
3
4
5
6
7
8

Figure 6. The multiple transcripts of *Cbr-hig-1* and *cni-hig-1* and the exon/intron structure. Transcripts sequences were obtained from Wormbase and GenBank. Predictions appear to be incomplete as *Cbr-hig-1* lack a start codon. A 5' extension of exon 5 is indicated in white.

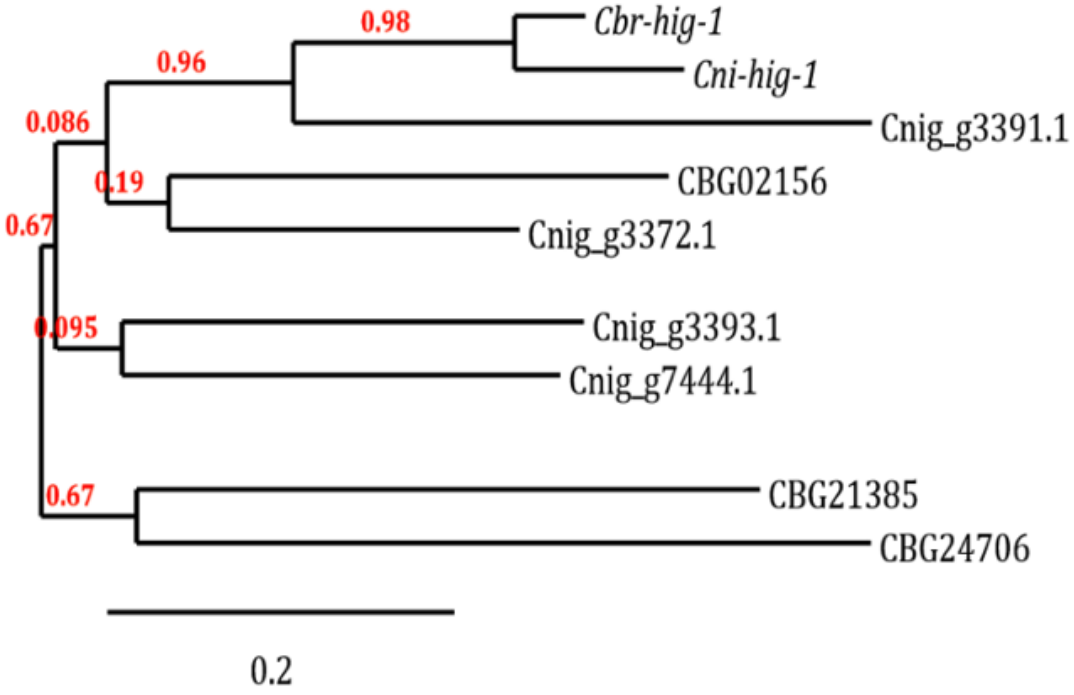

0.98
Cbr-hig-1
0.96
Cni-hig-1
0.086
Cnig_g3391.1
0.19
CBG02156
0.67
Cnig_g3372.1
0.095
Cnig_g3393.1
Cnig_g7444.1
0.67
CBG21385
CBG24706
0.2

Figure 7. ML phylogeny of *cbr- and cni-hig-1* paralogs based on inferred protein sequences between exons 4 and 7 with accompanying branch support aLRT values.

Discussion

Meiotic Silencing of Unpaired Chromosomes

The identification of *Cbr-hig-1* as a hybrid lethal gene supports the model of suppression of male specific lethality by *cbr-him-8* described in Ragavapuram et al., (2016). The meiotic silencing of mispaired X-chromosomes caused by *cbr-him-8* would have acted in a similar manner on the X-chromosome as *Cbr-hig-1* as the RNA interference. Both methods of X-chromosome suppression are mediated by RNA, MSUC mediated by piRNAs, and RNAi mediated by siRNAs. The males produced by *cbr-him-8* mutants animals and *Cbr-hig-1* silenced animals survived to adulthood because of the silencing of the portion of the X-chromosome that contains *Cbr-hig-1* which is at least partially responsible for the male specific hybrid lethality phenotype. Males surviving to adulthood produced by both *cbr-him-8* mutants and *Cbr-hig-1* suppressed animals are sterile.

The end result of both was the production of viable male hybrid progeny. It is likely that *Cbr-hig-1* was one of the X-linked genes identified by Ragavapuram et al., (2016) as a maternal effect suppressor of male specific lethality. In Ragavapuram et al., (2016) progeny from heterozygous *cbr-him-8* animals produced males at a low rate that is consistent with a recessive and maternal effect. The expected male progeny ratio for a recessive gene was eleven percent higher than what was observed in this study. There was nothing from the results of RNA interference of *Cbr-hig-1* that contested this finding. The rate of males produced from *Cbr-hig-1* silenced animals was 18% which is consistent with this finding. The co-suppression assay identified at least one additional hybrid lethal gene within the *C. briggsae* X-chromosome but the suppression of *Cbr-hig-1* can independently suppress male specific lethality.

Characterization of *Cbr-hig-1*

Male specific lethality was suppressed by the silencing of *Cbr-hig-1* by RNA interference. The mechanics of this process are not yet well understood. However, there are several clues to how *Cbr-hig-1* and *cni-hig-1* could be acting dysgenically to produce the hybrid lethal phenotype. The Incorporation of the ancestral hig-1 gene must have occurred in the most recent common ancestor of *C. briggsae* and *C. nigoni* as the syntenic orthologs are absent in any other *Caenorhabditis* species including *C.sinica* the next most closely related species. These orthologs do not provide any information as to the origin of the sequence of *Cbr-hig-1*. There are several paralogs of *Cbr-hig-1* but no one paralog was clearly identified as ancestral. Nor do sequence comparisons give any indication of gene function. The *Cbr-hig-1* sequence is homologous to some domains in some c-type lectins within *Caenorhabditis*, but CBR-HIG-1 does not contain the lectin-binding domain.

The inclusion of this ancestral hig-1 gene and subsequent divergence that lead to the development of a male specific hybrid lethal gene. The biggest clue as to how *Cbr-hig-1* accomplishes this is the region of the predicted protein structure that is identified as a von Willebrand type A proteins (vWA) domain which are involved in the assembly of large protein complexes. There are several differences within each of these genes that could be the culprit. Any of the several single nucleotide changes could amount the change needed to produce the dysgenic interaction. The changes in splice site usage and 5’ start sites are also good candidates that result in significant coding and structural changes. The same site that results in a 5’ extension of exon 5 in all *cni-hig-1* transcripts makes a differential splice site usage that creates the gap in alignment at the position of

helix 6. An edit at this site or any other could indicate whether or not that particular change is responsible for the dysgenic interactions.

Limitations

RNAi is unable to produce a heritable change in the genome so a strain featuring a knockout of *Cbr-hig-1* could not be constructed. A change in the sequence of *Cbr-hig-1* was attempted using Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR), however a successful knockout was not produced through the course of several successful injections. Information based on transcript and secondary protein structure where generated based on projections from known sequences and structures. These inferences will need to be confirmed using benchtop experiments.

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Appendix I. PCR Primers used in two-site CRISPR evaluation assay.

Primer	sequence	distance from cut site (bp)
239 50 R	atgaaatccatgcgagcatc	51
239 50 L	ttttcaaattcacttgattc	50
239 200 R	gaaccctatcggcaccgggt	202
239 200 L	cagaggagaaccaagtagaa	167
239 400 R	cttgagctgcaatcaatgca	402
239 400 L	atgagaattgcagaaagact	404
927 50 R	cacaaagcccaaaagcctaa	72
927 50 L	gatttttcgtcgaaaacagt	55
927 200 R	cgcatgaccaactgataagaa	213
927 200 L	ctgttacttgcgggaattca	208
927 400 R	ccaattattgctctcgcagc	400
927 400 L	ctccaacactcaagcacca	415